

## Cholesterol Orientation and Dynamics in Dimyristoylphosphatidylcholine Bilayers: A Solid State Deuterium NMR Analysis

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**ABSTRACT** Proton decoupled deuterium NMR spectra of oriented bilayers made of DMPC and 30 mol % deuterated cholesterol acquired at 76.8 MHz (30°C) have provided a set of very accurate quadrupolar splitting for eight C-D bonds of cholesterol. Due to the new precision of the experimental data, the original analysis by Dufourc et al. (1984. *Biochemistry*. 23:6062–6071) had to be reconsidered. We performed a systematic study of the influence on the precision and uniqueness of the data-fitting procedure of: (i) the coordinates derived from x-ray, neutron scattering, or force field-minimized structures, (ii) internal mobility, (iii) the axial symmetry hypothesis, and (iv) the knowledge of some quadrupolar splitting assignments. Good agreement between experiment and theory could be obtained only with the neutron scattering structure, for which both axial symmetry hypothesis and full order parameter matrix analysis gave satisfactory results. Finally, this work revealed an average orientation of cholesterol slightly different from those previously published and, most importantly, a molecular order parameter equal to  $0.95 \pm 0.01$ , instead of  $0.79 \pm 0.03$  previously found for the same system at 30°C. Temperature dependence in the 20–50°C range shows a constant average orientation and a monotonous decrease of cholesterol  $S_{\text{mol}}$ , with a slope of  $-0.0016 \text{ K}^{-1}$ . A molecular order parameter of  $0.89 \pm 0.01$  at 30°C was determined for a DMPC/16 mol % of cholesterol.

### INTRODUCTION

Cholesterol is a major component of plasma membrane, where it plays a reinforcing role. Its condensing effect on the lipid fatty acids was demonstrated by several techniques and authors (Worcester and Francks, 1976; Stockton and Smith, 1976; Delmelle et al., 1980; Deinum et al., 1988; McMullen et al., 1993; McMullen and McElhaney, 1996). Cholesterol-lipid interactions are responsible for these properties and depend on cholesterol structure, localization, orientation, and dynamics in the membrane. Cholesterol's vertical localization, studied largely by x-ray, neutron diffraction, and <sup>2</sup>H NMR, is such that its OH group is positioned in the vicinity of the phospholipid glycerol region and its fused ring system is near the fatty acids (Franks and Lieb, 1979; Worcester and Francks, 1976; Leonard and Dufourc, 1991; Dufourc et al., 1984, 1992; Douliez et al., 1995, 1996; Pott et al., 1995; Faure et al., 1996). Due to its amphipathic properties, cholesterol is oriented within a lipid bilayer with its long axis normal to the membrane surface. The polar hydroxyl group points toward the bilayer surface and the rigid nucleus interacts with the phospholipid acyl chains in order to optimize hydrophobic interactions.

In the early 1980s, Taylor et al. (1981) and Dufourc et al. (1984) determined by deuterium NMR the orientation and

molecular order parameter  $S_{\text{mol}}$  of cholesterol in egg PC and dimyristoylphosphatidylcholine (DMPC) bilayers. Their analysis was based on <sup>2</sup>H-NMR experiments of deuterated cholesterol embedded in unoriented membranes and calculations using x-ray coordinates of cholesterol derivatives. It was found that 50 mol % cholesterol had a  $S_{\text{mol}}$  of 0.87 in egg PC and 30 mol % cholesterol a  $S_{\text{mol}}$  of 0.80 in DMPC at room temperature. Their work has provided for many years a reference for orientation and dynamics of cholesterol and has been used for comparison with other techniques such as neutron scattering (Bloom and Bayerl, 1995; Douliez et al. 1996), NMR relaxation times analysis (Dufourc et al., 1992; Bonmatin et al., 1990), and molecular dynamics simulations (Gabdouline et al., 1996). Since then, significant methodological improvements in deuterium NMR of lipid bilayers have occurred, such as the use of oriented lipid bilayers (Jarrell et al., 1987) and proton decoupling (Jarrell et al., 1988). These methods allow the determination of quadrupolar splittings with a much higher resolution and accuracy than could be obtained using powder spectra. After performing such measurements with deuterated cholesterol, we realized that the original analysis had to be reconsidered because no correct agreement between experimental and calculated quadrupolar splittings could be obtained. Several possibilities were considered as possible sources of systematic error in the analysis, as discussed below: the insufficient quality of the x-ray structure of cholesterol used in the analysis, in particular concerning the coordinates of hydrogen atoms; the possible existence of internal mobility; the hypothesis of perfect axial symmetry; and ambiguities arising from unknown signs and assignments of the quadrupolar splittings.

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## MATERIALS AND METHODS

### Deuterated cholesterol

The  $\Delta^5$ -[2,2,3,4,4,6- $^2\text{H}_6$ ]cholesten-3 $\beta$ -ol was synthesized by a modification of a reported procedure (Gruenke and Craig, 1979) and the synthesis is detailed in the publication of Dufourc et al. (1984).

The  $\Delta^5$ -[6,7,7- $^2\text{H}_3$ ]cholesten-3 $\beta$ -ol was prepared according to the method of Goad et al. (1982), starting from 3 $\alpha$ ,5 $\alpha$ -cyclocholestan-6-one whose synthesis is detailed in the publication of Palmer et al. (1978). The  $\Delta^5$ -[6,7,7- $^2\text{H}_3$ ]cholesten-3-ol was characterized by electron impact mass spectrometry  $m/z$  as follows: 389[M] $^+$ , 374[M-CH $_3$ ] $^+$ , 371[M-H $_2$ O] $^+$ , 356[M-H $_2$ O-CH $_3$ ] $^+$ , 303, 275, 246; and by  $^1\text{H}$  NMR(CDCl $_3$ ) as follows:  $\delta$  0.72 (s, H-18), 0.89 (d, H-26 and H-27), 0.92 (d, H-21), 1.2 (s, H-19), 3.7 (m, H-3 $\alpha$ ).

### Oriented bilayers

Bilayers of oriented lipids were prepared by stacking 60 circular cover slips (diameter 0.5 cm and thickness 0.15 mm, Polylabo, Strasbourg, France) in a NMR Kelef tube (7-mm OD, 25 mm long). The normal to the plates was placed at 90 degrees with respect to the magnetic field. The cover slips were first pickled overnight in fuming nitric acid, then rinsed carefully and dried overnight at 40°C. The lipid mixture was then spread on the surface of each glass. It consisted of a solution of 70 mol % of DMPC (8.1 mg, Sygena Lipids, Inc.) and 30 mol % of deuterated cholesterol (2 mg) in 730  $\mu\text{l}$  of filtered 2-propanol (Teflon filters, 0.2  $\mu\text{m}$ , Millipore, Paris, France). The lipid surface density was 0.7 mg/cm $^2$ . After solvent evaporation under vacuum overnight, the plates were stacked into the NMR Kelef tube. The lipids were then hydrated at 37°C by equilibration overnight with deuterium-depleted vapor water (Aldrich Inc., St. Quentin Fallavier, France) and before the NMR analyses, 5  $\mu\text{l}$  of deuterium-depleted water were added in the tube to ensure excess humidity. Such a procedure ensures a bilayer plane orientation parallel to that of the plates. Two samples were prepared, one containing the  $\Delta^5$ -[2,2,3,4,4,6- $^2\text{H}_6$ ]cholesten-3 $\beta$ -ol and the other with the  $\Delta^5$ -[6,7,7- $^2\text{H}_3$ ]cholesten-3 $\beta$ -ol.

### $^2\text{H}$ NMR

Spectra were recorded at 30°C on a Bruker AMX500 spectrometer (Wissembourg, France), with proton decoupling in a solid state probe equipped with a solenoid coil doubly tuned for deuterium (76.77 MHz) and proton resonances. Quadrupolar echoes were obtained by using two 90° pulses of 3.6  $\mu\text{s}$  width with interpulse delay of 40  $\mu\text{s}$ , repetition rate of 1 s (to prevent excessive heating of the sample), and sweep width of 500 kHz; the number of scans was 20 K. Proton decoupling was performed during acquisition with a decoupling power of 20 kHz.

### Simulation of quadrupolar spectra and fitting to experimental data

Two approaches have been employed in order to simulate the experimental data (see Fig. 1 for definitions of angles and axes):

#### Axial symmetry

In the first approach, we assumed perfect axial symmetry of the C- $^2\text{H}$  segments' motions and followed the analysis of Dufourc et al. (1984). Briefly, the quadrupolar splitting of a particular C- $^2\text{H}_i$  bond is given by:

$$\Delta\nu_i = \frac{3}{2} \cdot A_Q \cdot S_{\text{bilayer}} \cdot S_{\text{mol}} \cdot S_{\text{loc}}^i \cdot \frac{3 \cos^2 \theta_i - 1}{2} \quad (1)$$

where  $A_Q$  is the static deuterium quadrupolar coupling constant (assumed to be 170 kHz for all C- $^2\text{H}_i$  bonds except the double bond in position 6, for

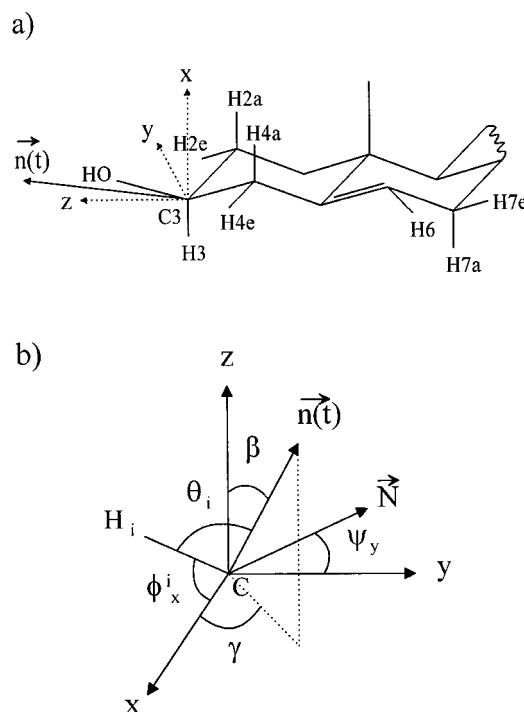


FIGURE 1 Representation of the axis system linked to the cholesterol molecule and of the various angles used in the calculations. (a) Rings A and B of cholesterol, numbering of labeled hydrogen atoms, and definition of the reference frame (C3, x, y, z) used in the simulations (same as in Dufourc et al., 1984). (b) Position in the reference frame of  $\vec{n}(t)$ , the instantaneous rotation axis of cholesterol, of  $\vec{N}$ , the bilayer normal ( $H_i$  is one of the eight labelled positions) and definitions of the angles ( $\beta, \gamma$ ), the polar coordinates of  $\vec{n}(t)$ ,  $\theta_i$  used in the axial symmetry hypothesis, and of angles  $\Phi_x^i$  and  $\psi_y$  used in the non axially symmetric treatment ( $\Phi_y^i, \Phi_z^i, \psi_x$ , and  $\psi_z$  are defined similarly).

which a value of 175 kHz was used, as in Dufourc et al. (1984));  $S_{\text{bilayer}}$  is the order parameter describing the degree of order and orientation of the bilayer normal ( $= -0.5$  in our case, i.e. for bilayers oriented at 90 degrees),  $S_{\text{mol}}$  is the molecular order parameter of cholesterol,  $S_{\text{loc}}^i$  is a local order parameter to account for internal motions within the cholesterol structure which may be estimated from molecular dynamics simulations ( $S_{\text{loc}}^i = 1$ , if the ring system is assumed to be rigid), and  $\theta_i$  is the angle between the C- $^2\text{H}_i$  bond and the axis of motion. Given the coordinates ( $l_i, m_i, n_i$ ) of the C- $^2\text{H}_i$  bond in a reference frame (C3, x, y, z) linked to the cholesterol rigid backbone, chosen as in Dufourc et al. (1984),  $\theta_i$  is given by:

$$\cos \theta_i = (l_i \cdot \cos \gamma \cdot \sin \beta + m_i \cdot \sin \gamma \cdot \sin \beta + n_i \cdot \cos \beta) / \sqrt{l_i^2 + m_i^2 + n_i^2} \quad (2)$$

where  $\beta$  and  $\gamma$  are the polar coordinates of the symmetry axis of motion in the reference frame. In this approach, ( $l_i, m_i, n_i$ ) are taken from a structure of cholesterol which may be determined by x-ray diffraction (Dufourc et al. used the structure determined by Sawsik and Craven (1980)), or other means which will be discussed later, and the unknowns are therefore  $\gamma, \beta$ , and  $S_{\text{mol}}$ . From the determination of several quadrupolar splittings in a rigid molecule, one can thus extract these numbers.

For a given cholesterol structure, coordinates of C- $^2\text{H}_i$  vectors were extracted from the corresponding file of atomic coordinates and used to calculate  $\cos \theta_i$  values for all eight C- $^2\text{H}_i$  vectors for which coupling constants were measured. For each pair of values ( $\beta, \gamma$ ), which were varied systematically in 1° increments, we calculated theoretical quadrupolar splittings, ranked them by increasing order, normalized them by dividing

by their highest value, and compared them with experimental values ranked and normalized in an identical way. This normalization was performed to eliminate the  $S_{\text{mol}}$  value, which by definition is the same for all the C-<sup>2</sup>H bonds linked to the rigid fused ring system. The output was filtered according to two criteria, the first of which is the maximum allowed error  $d_0$ : for all  $i$ ,  $d_i < d_0$ , where  $d_i = [R_i^{\text{calc}} - R_i^{\text{exp}}]^2$ , with  $R_i^{\text{exp}} = |(\Delta\nu_Q)_i^{\text{exp}}|$ , and  $R_i^{\text{calc}} = |(\Delta\nu_Q)_i^{\text{calc}}|/(\Delta\nu_Q)_{\text{max}}^{\text{calc}}$ . The second sorting criterium is the average error, RMSD, defined as  $1/(n-1)\sqrt{\sum_{i=1}^{n-1} d_i}$ ,  $n$  being the number of quadrupolar splittings measured (8 in our case). The average is calculated by dividing by  $(n-1)$  because one value of  $d_i$  is equal to zero. After selection of acceptable solutions for  $(\beta, \gamma)$ , the assignment,  $d_{\text{max}}$  (the largest value of  $d_i$ ,  $d_{\text{max}} < d_0$ ), the RMSD, and  $S_{\text{mol}}$  are calculated. The possibility is offered to specify some assignments and local order parameters  $S_{\text{loc}}^i$  when these are known.

A total of six structures were explored in this way: three structures of cholesterol analogs determined by x-ray crystallography, one structure determined by neutron diffraction, and two structures minimized with different force fields (CFF91 and CHARMM). A program performing this task has been developed in our laboratory in C and runs on a Silicon Graphics Indy workstation (and standard UNIX platforms) and is available from the authors.

### No axial symmetry

The second approach, which did not rely on the assumption of axial symmetry, was based on the full order parameter matrix analysis. It can be shown that the expression describing the quadrupolar line splitting  $\Delta\nu$  is given by:

$$\Delta\nu_i = \frac{3}{2} A_Q S_{\text{bilayer}} \cdot \left\{ A_{zz}^i S_{zz} + \frac{1}{3} (A_{xx}^i - A_{yy}^i) (S_{xx} - S_{yy}) + \frac{4}{3} (A_{xy}^i S_{xy} + A_{xz}^i S_{xz} + A_{yz}^i S_{yz}) \right\} \quad (3)$$

(Emsley, 1985; Sanders and Prestegard, 1991; Hong et al., 1995). The geometric factors  $A_{kl}^i$  ( $k, l = x, y, z$ ) can be computed in the chosen molecular frame of coordinates as:

$$A_{kl}^i = \frac{3 \cos \phi_k^i \cos \phi_l^i - \delta_{kl}}{2} \quad (4)$$

where  $\cos(\phi_k^i)$  are direction cosines specifying the orientation of a C-<sup>2</sup>H<sub>i</sub> bond with respect to the molecular fixed frame, and  $\delta_{kl}$  is the Kronecker symbol ( $\delta_{kl}$  equals 1 if  $k = l$  and 0 otherwise).  $S_{kl}$  are molecular order parameters describing the average order of molecular axes with respect to the bilayer normal. They are given by

$$S_{kl} = \left\langle \frac{3 \cos \psi_k \cos \psi_l - \delta_{kl}}{2} \right\rangle \quad (5)$$

where  $\psi_k$ , ( $k = x, y, z$ ) characterize the orientation of the molecular frame with respect to the bilayer normal. Using the fact that the order parameter matrix is traceless (Emsley, 1985), we can rewrite Eq. 5 in the following form:

$$\Delta\nu_i = -\frac{3}{4} A_Q \sum_{p=1}^5 a_p \Phi_p^i \quad (6)$$

to which the generalized least squares analysis (Press et al., 1992) is easily applicable. The unknown parameters  $a_p$  and the base functions  $\Phi_p^i$  are defined as follows:

$$a_1 = S_{xx} \quad \Phi_1^i = \frac{1}{3} (A_{xx}^i - A_{yy}^i) - A_{zz}^i$$

$$a_2 = S_{yy} \quad \Phi_2^i = \frac{1}{3} (A_{yy}^i - A_{xx}^i) - A_{zz}^i$$

$$a_3 = S_{xy} \quad \Phi_3^i = \frac{4}{3} A_{xy}^i$$

$$a_4 = S_{xz} \quad \Phi_4^i = \frac{4}{3} A_{xz}^i$$

$$a_5 = S_{yz} \quad \Phi_5^i = \frac{4}{3} A_{yz}^i$$

For a given cholesterol structure, coordinates of C-<sup>2</sup>H vectors were extracted from the corresponding file of atomic coordinates and used to calculate direction cosines and geometric factors  $A_{ij}$  for all of the eight C-<sup>2</sup>H vectors for which quadrupolar splittings were measured. Next, for a set of accepted assignments,  $\Delta\nu_i$  were computed. Finally, differences between calculated and observed quadrupolar splittings were used to assess the error level for this particular configuration. Because only the absolute values of the quadrupolar splittings are measured, there remains an ambiguity about the sign of each coupling. Therefore all sign combinations were tested, which for 8 C-<sup>2</sup>H vectors resulted in  $2^8 = 256$  variants for each assignment. However, because few of the assignments are certain, we decided to explore all the possibilities systematically. The total number of variants due to different assignments is  $8! = 40,320$ , which, combined with 256 sign options, gives a total of 10,321,920 configurations tested for each structure. A total of four structures were explored in this way: three cholesterol structures minimized with different force fields (CHARMM, CFF91, and ESFF) and a structure resulting from a neutron scattering study. The program performing this task has been developed in our laboratory. It has been written in Fortran 77 and runs on a Silicon Graphics Indy workstation. The source code is available from the authors.

## RESULTS

### <sup>2</sup>H NMR data

To determine the cholesterol reorientation axis in DMPC bilayers, two deuterated molecules were embedded in oriented bilayers: <sup>2</sup>H NMR spectra of the 90°-oriented bilayer containing either [6,7,7-<sup>2</sup>H<sub>3</sub>]cholesterol or [2,2,3,4,4,6-<sup>2</sup>H<sub>6</sub>]cholesterol are represented in Fig. 2. The use of the oriented bilayers and proton decoupling during acquisition provided an important reduction in linewidth and a much better resolution, as compared with standard <sup>2</sup>H NMR powder spectra obtained with liposomes (compare Fig. 2 in this paper with Fig. 3 in Dufourc et al., 1984). All six individual quadrupolar splittings could thus be resolved with [2,2,3,4,4,6-<sup>2</sup>H<sub>6</sub>]cholesterol, and a total of eight values were obtained using both sterols (Table 1). These quadrupolar splittings could be measured with a relative precision of <2% for the smallest quadrupolar splitting and <1% for the others, considering the digital resolution (30 Hz/Pt), the linewidth (500 Hz), and the good signal-to-noise ratio. Furthermore, six independent measurements were realized at 30°C on three separate samples and the quadrupolar splittings were found to be within these ranges in every case. These experimental errors were used as a basis for determining agreement between the simulated and experimental data. Since both sterols had deuterium in position 6, they provided a direct proof for the assignment of this

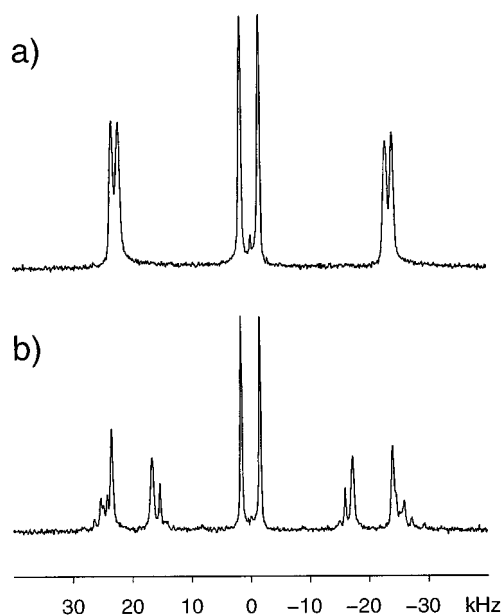


FIGURE 2 Proton decoupled,  $^2\text{H}$  NMR spectra of 90 degrees oriented bilayers of DMPC containing 30 mol % of deuterated cholesterol. (a) Lower spectrum,  $\Delta^5$ -[2,2,3,4,4,6- $^2\text{H}_6$ ]cholesten-3 $\beta$ -ol. (b) Upper spectrum,  $\Delta^5$ -[6,7,7- $^2\text{H}_3$ ]cholesten-3 $\beta$ -ol.

position and a way of ensuring that both spectra were acquired at identical angles and hydrations, thus giving the same quadrupolar splitting for position 6. In the two spectra of Fig. 2 *a* and 2 *b*, the two deuterons in position 6 are clearly assigned to the smallest  $\Delta\nu_Q$ , showing that position 6 is almost at magic angle  $54.7^\circ$  with respect to the molecular rotation axis. The axial deuterons (2a, 4a, 3) are almost parallel and form a similar angle with the rotation axis of cholesterol. Equatorial deuterons should form a second group, and finally there remain two deuterons in position 7 that display large quadrupolar splittings as shown in Fig. 2 *b* and Table 1.

### Analysis with the axial symmetry hypothesis at $30^\circ\text{C}$

This hypothesis is classically made for lipid bilayers in the liquid crystalline phase, in agreement with the observation of experimental spectra which present lineshapes typical of axially symmetric movements. This symmetry arises from the fast axial diffusion of the molecules (phospholipids and

cholesterol) about the bilayer normal. This axis of diffusion has been confirmed by examination of the angular dependence of quadrupolar splittings as a function of the bilayer orientation (Jarrell, 1987).

First, we analyzed the data assuming that  $S_{\text{loc}}^i = 1$ , i.e. without internal mobility within the four rings of cholesterol. For each set of coordinates, we selected the best pair of  $\beta, \gamma$  angles, and we evaluated the agreement between simulated and experimental spectra, which was reflected by two parameters:  $\sqrt{d_{\text{max}}}$ , the maximum error, and RMSD, which represents the average error over the eight quadrupolar splittings. We first performed these calculations without any assumption concerning the assignments (Table 2). Because we knew from our experiment the assignment of deuterons 6, 7a, and 7e (with a possible permutation between the last two), we then selected the subset of solutions which were in agreement with the known assignments (Table 3). It clearly appears that no correct solution can be found on the basis of the x-ray structure originally used by Dufourc et al. (1984). Indeed, although an approximate solution may be found without assignments (RMSD = 4.7%), when imposing an assignment compatible with our data, the RMSD (19%) and the maximum error (36%) are not acceptable. Because we suspected that the poor accuracy of hydrogen coordinates derived from the x-ray structure could be one cause of this discrepancy, we searched for high-precision structures of cholesterol analogs (which do not differ from cholesterol at the level of rings A, B, and C) in the Cambridge crystallographic database (Allen et al., 1983). RX-1983 (see Table 2 for the definitions of these structure names) and RX-1994 were among the highest-precision structures (lowest R factor, low temperature x-ray diffraction) that we found. It can be seen from Tables 2 and 3 that although the results did improve significantly (maximum error of 7.9%, RMSD of 4.4% for RX-1994, with correct assignments), we were still far above experimental precision. Coordinates derived from molecular mechanics, using two different force fields (cff91, the force field developed by MSI Inc. (Discover), and CHARMM, the standard force field developed by M. Karplus) were also tested; it is apparent from Tables 2 and 3 that the results are not any better.

We finally tested a structure of a cholesterol analog, called neutrons in Tables 2 and 3, which was determined by neutron diffraction at low temperature (123 K, McMullan et al., 1992). Because the scattering length of hydrogen in

TABLE 1 Quadrupolar splittings for cholesterol C- $^2\text{H}$  bonds in DMPC membranes

		$\Delta\nu_Q$ (kHz)					
Known assignments*	6	7e or 7a		7e or 7a			
30 mol % cholesterol	3.22	31.34, 33.93	45.74	47.49	48.06	50.84	53.65
16 mol % cholesterol	2.27	30.19, 31.42		45.19		48.61	n.r.

Bilayer normal oriented at  $90^\circ$  with respect to the magnetic field;  $T = 30^\circ\text{C}$ . n.r., not resolved.

\*The partial assignment here is based on the comparison between the spectra obtained with the two labeled cholesterol.



**TABLE 2** Analysis of six different cholesterol molecular structures within the axial symmetry hypothesis

Structures	$\gamma$ (°)	$\beta$ (°)	$S_{\text{mol}} \pm 0.01$	$\sqrt{d_{\text{max}}}$ (%)	RMSD (%)	Assignment
	°					
RX-1980*	70	37	0.86	8.0	4.7	7e,2a,4a,7a,2e,3,4e,6
RX-1983 <sup>#</sup>	40	19	0.89	3.7	2.2	2e,7e,6,3,4e,7a,4a,2a
RX-1994 <sup>§</sup>	228	10	0.92	7.7	4.2	4e,2e,6,7e,3,2a,4a,7a
Neutrons <sup>¶</sup>	9	11	0.96	2.4	1.1	6,4e,2e,4a,7e,7a,2a,3
MM-cff91 <sup>  </sup>	12	10	1.01	3.0	1.8	6,4e,2e,7e,4a,2a,7a,3
MM-CHARMM <sup>  </sup>	121	23	0.96	6.1	2.6	7e,6,2e,4e,3,4a,2a,7a

Calculations performed without  $S_{\text{loc}}^i$  (i.e. internal mobility) and without restrictions on the assignments.

$S_{\text{mol}}$ , molecular order parameter;  $\sqrt{d_{\text{max}}}$ , maximum error; RMSD, mean error between experimental data and theoretical values (defined in Materials and Methods); assignment, each number corresponds to a specific deuterium as shown in Fig. 1, by order of increasing  $\Delta\nu_Q$ . This assignment is an output of the simulation, which explored systematically all possible assignments.

\*RX-1980: cholesterol laurate (Sawicki and Craven, 1980); x-ray structure used in Dufourc et al. (1984).

<sup>#</sup>RX-1983: 17 $\alpha$ -phenyl-androst-5-ene-3 $\beta$ ,17 $\beta$ -diol (Weeks et al., 1983), x-ray structure.

<sup>§</sup>RX-1994: 17 $\alpha$ -benzyl-5-androst-5-ene-3 $\beta$ ,17 $\beta$ -diol (Stankovic et al., 1994), x-ray structure.

<sup>¶</sup>Neutrons: [20-CD<sub>3</sub>]-methylpregnene-3 $\beta$ ,20-diol (McMullen et al., 1992), structure obtained by neutron diffraction at 123 K.

<sup>||</sup>MM-cff91 and MM-CHARMM: structures obtained by minimization of the RX-1980 structure using the force field cff91 (present in the Insight molecular modeling package, MSI, Inc., Orsay, France), and the charmm force field (standard force field developed by M. Karplus).

neutron scattering experiments is comparable in magnitude to that of heavy atoms, hydrogen positions are expected to be determined with much better accuracy by neutron than by x-ray diffraction, and therefore the C-<sup>2</sup>H<sub>i</sub> vector coordinates are expected to be very accurate. Tables 2 and 3 show that indeed the agreement between experiment and simulation is then excellent; the rmsd (1.2%) is below the experimental error. It should be stressed that, only in this case, the best solution in terms of rmsd gave almost the correct assignment (actually with a small inversion between deuterium 4a and 7e, which have very close quadrupolar splittings, and with very similar values for  $\beta$  and  $\gamma$  angles).

The introduction of internal mobility did not improve the fittings.  $S_{\text{loc}}^i$  were estimated from molecular dynamics simulation of cholesterol in vacuo, using both cff91 and CHARMM force fields. However, in both cases,  $S_{\text{loc}}^i < 0.94$  were found, which is not compatible with the value  $S_{\text{mol}} \cdot S_{\text{loc}}^i = 0.96$  obtained in Table 3, because it would imply  $S_{\text{mol}} > 1$ . Such low  $S_{\text{loc}}^i$  value may be due to the fact that the simulations were performed in vacuo. We did not continue our analysis in this direction because the experimental accuracy was not sufficient to discriminate among several models of internal mobility.

We have also tested, at the same temperature of 30°C, DMPC bilayers containing 16 mol % of [2,2,3,4,4,6-

<sup>2</sup>H<sub>6</sub>]cholesterol instead of 30 mol %. When the amount of cholesterol was reduced by half, all the quadrupolar splittings of the C-<sup>2</sup>H link obtained were smaller (Table 1). The results obtained with the neutrons structure were close to those with 30 mol % cholesterol, i.e., ( $\gamma$ ,  $\beta$ ) = (10°, 11°), an identical assignment, and a smaller  $S_{\text{mol}}$ , shifting from 0.96 with 30 mol % of cholesterol to 0.89 with 16 mol % and showing, as expected, a higher mobility with a lower amount of cholesterol.

### Temperature dependence

Temperature dependence of the spectra was investigated between 10°C and 50°C. At 10°C a drastic loss of resolution and sensitivity was observed, as was already noted on powder spectra of DMPC-30 mol % cholesterol (Dufourc et al., 1984). This loss of axial symmetry in the spectrum corresponds to a phase transition to the L<sub>OB</sub> phase (liquid ordered gel-like phase), which has been observed to be completed below 25°C for a DPPC-30 mol % cholesterol bilayer (McMullen et al., 1995) and may be estimated to be completed below 10–15°C for a DMPC-30 mol % cholesterol bilayer (McMullen et al., 1993). Between 10°C and 50°C spectra similar to those of Fig. 2 have been observed,

**TABLE 3** Analysis of six different cholesterol molecular structures within the axial symmetry hypothesis and a restriction on the assignment of deuterons 6 and 7

Structures	$\gamma$ (°)	$\beta$ (°)	$S_{\text{mol}} \pm 0.01$	$\sqrt{d_{\text{max}}}$ (%)	RMSD (%)	Assignment (6, 7e, and 7a imposed)
	°					
RX-1980*	132	16	0.82	36	19	6,2e,4e,7e,3,7a,2a,4a
RX-1983 <sup>#</sup>	2	14	0.87	8.9	5.7	6,2e,4e,7e,3,7a,4a,2a
RX-1994 <sup>§</sup>	350	8	1.00	7.9	4.4	6,2e,4e,7e,4a,7a,2a,3
Neutrons <sup>¶</sup>	11	11	0.96	2.1	1.2	6,4e,2e,7e,4a,7a,2a,3
MM-cff91 <sup>  </sup>	161	29	1.80	18	13	6,2e,4e,7e,3,7a,2a,4a
MM-CHARMM <sup>  </sup>	156	5	0.92	24	14	6,2e,4e,7e,3,7a,2a,4a

For legend, see Table 2.

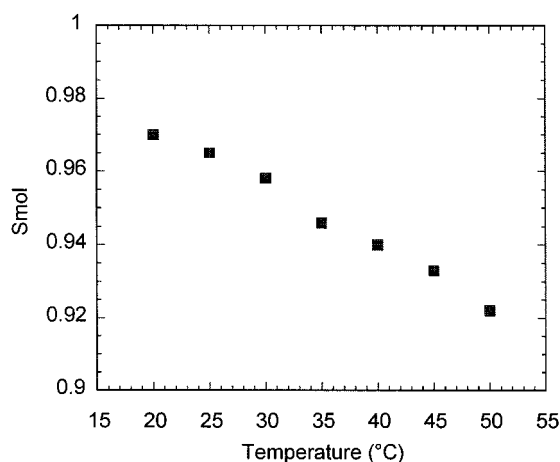


FIGURE 3 Evolution of the molecular order parameter of cholesterol  $S_{mol}$  as a function of temperature, for bilayers made of DMPC-30 mol % cholesterol.

with a monotonous reduction of the quadrupolar splittings when the temperature is increased. Analysis of the data within the axial symmetry hypothesis and using the neutron structure showed no modification of the average orientation of cholesterol over the whole temperature range (constant values for  $\gamma$  and  $\beta$ ) and a monotonous decrease of  $S_{mol}$  from 0.97 (20°C) to 0.92 (50°C), as illustrated in Fig. 3. According to the temperature composition diagram of a DMPC/cholesterol mixture (McMullen et al., 1993, 1995), this temperature range spans the entire coexistence region of  $L_{O\alpha}$  (liquid ordered liquid-crystalline) and  $L_{O\beta}$  phases. As seen from deuterium NMR (microsecond time scale of averaging), one population of cholesterol molecules is observed, and it has a highly restricted wobbling motion over this temperature range.

### Analysis without axial symmetry assumption at 30°C

The axial symmetry hypothesis, which is often used in the analysis of lipid bilayers in the fluid phase, could seldom be verified experimentally. Our data provided an ideal way of doing it.

Results of the calculations (performed as described in Materials and Methods) were filtered according to two criteria. First, the error between the observed and generated quadrupolar splittings should be below the level of experimental error ( $\sim 2\%$ ). Secondly, the solution should be physically acceptable, i.e., the values of order parameters determined from numerical analysis should be contained in the interval  $(-0.5; 1)$ . Very few results satisfied these two criteria simultaneously, and it was clear from the analysis of the results that, again, only the structure obtained from neutron scattering data was capable of producing coupling patterns close to those observed experimentally. Other structures gave results with errors larger than 10%, i.e., much larger than that experimentally observed ( $\sim 2\%$ ). The

order parameter matrix could be diagonalized to obtain the order parameters of the principal molecular axes with respect to the bilayer normal. The best result found (error  $\approx 0.96\%$ ) yielded the following values of the diagonalized order parameter matrix:

$$(S_{xx}, S_{yy}, S_{zz}) = (-0.48, -0.46, 0.94),$$

from which it follows that the asymmetry parameter  $\eta = 0.02$ , indicating that the studied system has a nearly perfect axial symmetry, within experimental accuracy. Moreover, the orientation of the molecular fixed frame with respect to the principal axes can be deduced from the data (see Fig. 2). The two angles  $\gamma$  and  $\beta$  were found to be  $7.4^\circ$  and  $12.0^\circ$ , respectively.

## DISCUSSION

The two approaches, with and without axial symmetry assumption, were thus in excellent agreement, with  $S_{mol} = 0.96$  (axial) and 0.94 (no axial symmetry), and  $(\gamma, \beta) = (11, 11)$  (axial) and  $(7, 12)$  (no axial symmetry).

Assuming axial symmetry, it was possible to generate data showing the dependence of RMSD error on these two angles. Thus obtained, sparse data were fed to another program, which generated contours by means of Voronoi tessellation algorithm (Watson, 1982). The results are shown in Figs. 4 and 5. Fig. 4 *a* presents the RMSD error level contours in the general case, in which no restrictions on assignments have been imposed. It can be seen that several regions exist in which errors are relatively low and which correspond to different molecular frame orientations. There exists, however, only one region in which RMSD errors approach the experimentally observed error. This region is shown in detail in Fig. 4 *b* and corresponds to the best solution found in our analysis. It shows that for an experimental accuracy of 2%, the precision on the angle  $\gamma$  and  $\beta$  which characterize the cholesterol orientation is  $\pm 1^\circ$  for  $\beta$  and  $\pm 5^\circ$  for  $\gamma$ .

Fig. 5 shows the error level contours obtained the same way as those in Fig. 4 *a*, but with the assignment of the vector involving deuterium in position 6 fixed, and assignments of positions 7a and 7e constrained to an interchange between these two nuclei only. With these restrictions, the plot shows that only a subset of regions is acceptable. Our systematic study highlighted the following points about determining orientation and order parameter of a molecule in a membrane by using this approach:

- The coordinates used in the analysis play a crucial role and must be very precise. This is an important limitation of the method, because neutron diffraction structures may not always be available. Structures of cholesterol obtained by molecular mechanics are not satisfactory in this respect, and our work reveals that current force fields need to be further optimized with respect to hydrogen atom positions. It can be mentioned here that one could have combined solid state NMR data with x-rays, if one

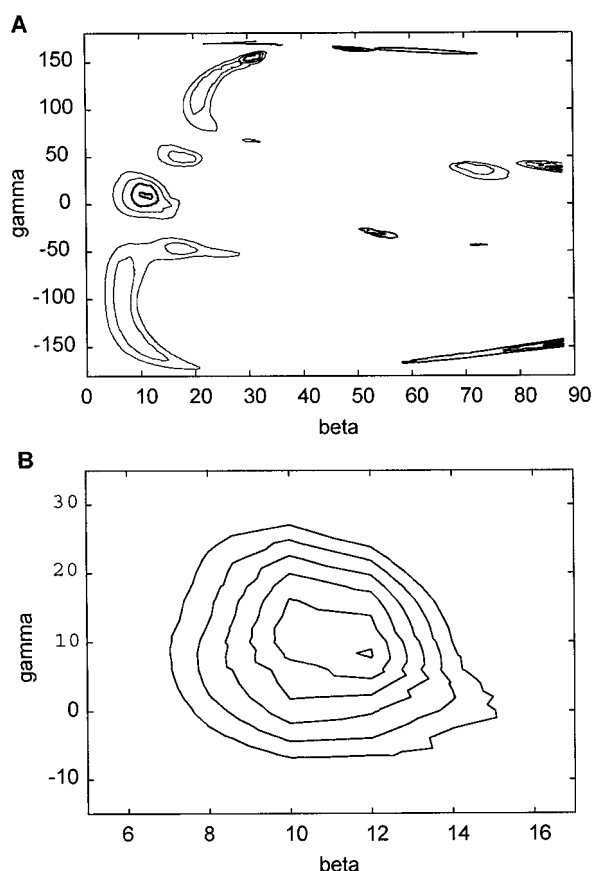


FIGURE 4 Influence of the error level on the uniqueness of data analysis. Using the accurate neutron cholesterol structure, we plotted RMSD contour plots as a function of the angles  $\beta, \gamma$ , without assignment of the spectrum. (a) whole range of  $\beta, \gamma$  values: contours at RMSD 2.5% and 5% (thick lines), 7.5% and 10% (thin lines). (b) enlargement of the area of minimum RMSD: RMSD at 1%, 2%, 3%, 4%, 5%, and 6%.

would have worked on the carbons skeleton, i.e., used the very accurate carbon coordinates and dipolar  $^{13}\text{C}$ - $^{13}\text{C}$  NMR interaction (Sanders and Prestegard, 1991) on a  $^{13}\text{C}$ -labeled cholesterol molecule.

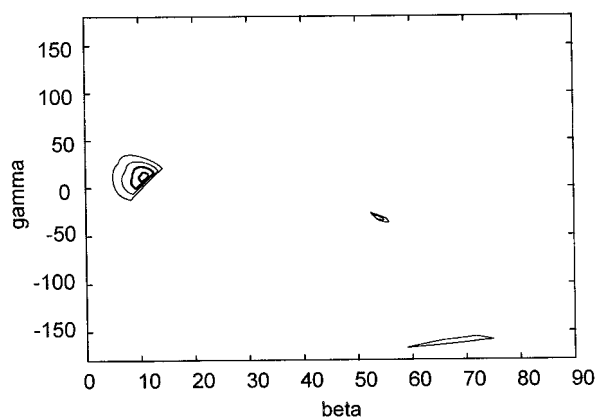


FIGURE 5 Same as Fig. 4 a, but with restriction on the assignment of deuterons 6 and 7.

- If the coordinates are not very accurate and/or the experimental data are not very precise, it is essential to obtain the deuteron assignments from independent measurements. Otherwise, the problem is underdetermined (see Fig. 4). In this respect, new methods for assigning deuterium NMR spectra are of utmost importance (Auger et al., 1997).
- The axial symmetry hypothesis could be safely used to interpret the data.
- Internal mobility did not need to be included in the calculations at the level of precision (2%) at which we were analyzing the data. If it is present, it has to be of low amplitude because a global order parameter  $S_{\text{mol}}$  of  $0.96 \pm 0.01$  was found ( $S_{zz} = 0.94 \pm 0.01$  without axial symmetry).

Using different structural data, namely x-ray coordinates of cholesterol laurate, Taylor and Dufourc (Taylor et al. 1981; Dufourc et al. 1984; Léonard and Dufourc, 1991) reported molecular order parameters ranging from 0.6 to 0.9 depending on cholesterol concentration, lipid nature, and temperature. Interestingly, it appears that the molecular ordering does not change much with temperature when cholesterol concentrations in phospholipids are  $\geq 30$  mol % (Léonard and Dufourc, 1991). To compare with the present results, it is interesting to quote some values. Taylor and coworkers reported  $S_{\text{mol}} = 0.89$  for 40 mol % cholesterol in egg PC at room temperature, whereas Dufourc and coworkers quoted  $S_{\text{mol}} = 0.8$  for 30 or 50 mol % in DMPC. Weisz et al. (1992) reported  $S_{\text{mol}} = 0.85$  for 40 mol % cholesterol in DMPC at ambient temperature. All these values are markedly lower than what we found for 30 mol % cholesterol in DMPC. Deviations range between 5–17% and are clearly due to the intrinsic inaccuracy of the proton x-ray coordinates and NMR data, as shown in the Results section.

It is interesting at this level of the discussion to comment on the implications of a cholesterol order parameter greater than that determined up to now. The molecular order parameter is an accurate description of the so-called wobbling in a cone of rigid moieties. It is clear from the above that the dynamics of cholesterol appears to be adequately described by an anisotropic rapid diffusion in a cone of semi-angle of only a few degrees. Quantitative values for this semi-angle depend very much on the nature and strength of the orienting potential. But it is clear that a drastic reduction can be predicted when  $S_{\text{mol}}$  goes from 0.8 to 0.94. Thus, the amplitude of cholesterol wobbling is very small indeed.

Order parameters are also used in calculating the thickness of lipid bilayers. Recently, Douliez et al. (1995) reported that the average length of a hydrocarbon phospholipid chain could be calculated from measurement of intramolecular and intermolecular (also simply called molecular) order parameters. In their Eq. 18,  $S_{\text{mol}}$  appears in the term  $(1 + \sqrt{1 + 8S_{\text{mol}}})/4$ . Changing  $S_{\text{mol}}$  from 0.8 to 0.94 leads to an increase in the estimated hydrophobic length of each hydrocarbon chain by 5%, i.e., 0.5–0.7 Å. In their study the authors also reported that a  $S_{\text{mol}}$  variation

from 0.7 to 1 was leading to an increased estimation of the number of gauche defects per chain by 30–60%. Changing  $S_{\text{mol}}$  from 0.8 to 0.94 will thus increase the estimated number of gauche defects by 15–30%.

Finally, the molecular order parameter and the average orientation of cholesterol in a DMPC bilayer, determined by Taylor et al. in 1981, have been used several times in quantitative analysis of NMR relaxation times in order to extract correlation times for the various movements present in a bilayer (Mayer, 1990; Weisz, 1992). Although the molecular order parameters of cholesterol and phospholipids may not necessarily be the same, our new data may necessitate modification of the results of such analyses to an extent which remains to be determined.

## CONCLUSION

A restatement of cholesterol ordering in biomembranes has been proposed and several computer programs have been developed to facilitate the analysis of quadrupolar splitting in an oriented sample and the extraction of molecular order parameters. These findings contribute to a more accurate description of molecular ordering of cholesterol in one specific model membrane system. In order to fully understand the mechanism of cholesterol-lipid interactions and the biological role of cholesterol, the strategy developed herein should now be extended to a variety of model biomembranes, considering different cholesterol concentration, fatty acyl length and degree of unsaturation, polar headgroups, and several natural lipid mixtures, in order to account for the great diversity of lipids found in natural membranes.

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## REFERENCES

- Allen, F. H., O. Kennard, and R. Taylor. 1983. Systematic analysis of structural data as a research technique in organic chemistry. *Acc. Chem. Res.* 16:146–153.
- Auger, C., A. Lesage, S. Caldarelli, P. Hodgkinson, and L. Emsley. 1997. Deuterium—carbon NMR correlation spectroscopy in oriented materials. *J. Am. Chem. Soc.* 119:12000–12001.
- Baenziger, J. E., I. C. P. Smith, R. J. Hill, and H. C. Jarrell. 1988. Direct measurement of deuterium-deuterium dipolar coupling and analysis of the ordering of a specifically deuterated diunsaturated lipid. *J. Am. Chem. Soc.* 110:8229–8231.
- Bloom, M., and T. M. Bayerl. 1995. Membranes studied using neutron scattering and NMR. *Can. J. Phys.* 73:687–696.
- Bonmatin, J. M., I. C. P. Smith, H. C. Jarrell, and D. J. Siminovitch. 1990. Use of a comprehensive approach to molecular dynamics in ordered lipid systems: cholesterol reorientation in oriented lipid bilayers. A  $^2\text{H}$  NMR relaxation case study. *J. Am. Chem. Soc.* 112:1697–1704.
- Deinum, G., H. van Langen, G. van Ginkel, and Y. K. Levine. 1988. Molecular order and dynamics in planar lipid bilayers: effects of unsaturation and sterols. *Biochemistry*. 27:852–860.
- Demelle, M., K. W. Butler, and I. C. P. Smith. 1980. Saturation transfer electron spin resonance spectroscopy as a probe of anisotropic motion in model membrane systems. *Biochemistry*. 19:698–704.
- Douliez, J. P., A. Leonard and E. J. Dufourc. 1995. Restatement of order parameters in biomembranes: calculation of C-C bond order parameters from C-D quadrupolar splittings. *Biophys. J.* 68:1727–1739.
- Douliez, J. P., A. Léonard, and E. J. Dufourc. 1996. Conformational order of DMPC sn-1 versus sn-2 chains and membrane thickness: an approach to molecular protrusion by solid state  $^2\text{H}$ -NMR and neutron diffraction. *J. Phys. Chem.* 100:18450–18457.
- Dufourc, E. J., C. Mayer, J. Strohrer, G. Althoff, and G. Kothe. 1992. Dynamics of phosphate head groups in biomembranes: comprehensive analysis using phosphorus-31 nuclear magnetic resonance lineshape and relaxation time measurements. *Biophys. J.* 61:42–57.
- Dufourc, E. J., E. J. Parish, S. Chitrakorn, and I. C. P. Smith. 1984. Structural and dynamical details of cholesterol-lipid interaction as revealed by deuterium NMR. *Biochemistry*. 23:6062–6071.
- Emsley, J. W. 1985. NMR of liquid crystals. In NATO series, D. Reidel, Dordrecht.
- Faure C., J. F. Tranchant, and E. J. Dufourc. 1996. Comparative effects of cholesterol and cholesterol sulfate on hydration and ordering of dimyristoylphosphatidylcholine membranes. *Biophys. J.* 70:1380–1390.
- Franks, N. P., and W. R. Lieb. 1979. The structure of lipid bilayers and the effects of general anaesthetics. An X-ray and neutron diffraction study. *J. Mol. Biol.* 133:469–500.
- Gabdoulline R. R., G. Vanderkooi, and C. Zheng. 1996. Comparison of the structure of dimyristoylphosphatidylcholine in the presence and in the absence of cholesterol by molecular dynamics simulations. *J. Phys. Chem.* 100:15942–15946.
- Goad, L. J., M. A. Breen, N. B. Rendell, M. E. Rose, and A. P. Wade. 1982. Synthesis of deuterium labeled cholesterol and steroids and their use for metabolic studies. *Lipids*. 17:982–991.
- Gruenke, L. D., and J. C. Craig. 1979. *J. Labeled Compd. Radiopharm.* 16:495–499.
- Hong, M., K. Schmidt-Rohr, and D. Nanz. 1995. Study of phospholipid structure by  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{31}\text{P}$  dipolar couplings from two-dimensional NMR. *Biophys. J.* 69:1939–1950.
- Jarrell, H. C., P. A. Jovall, J. B. Giziewicz, L. A. Turner, and I. C. P. Smith. 1987. Determination of conformational properties of glycolipid head groups by  $^2\text{H}$  NMR of oriented multibilayers. *Biochemistry*. 26:1805–1811.
- Leonard, A., and E. J. Dufourc. 1991. Interactions of cholesterol with the membrane lipid matrix: a solid state NMR approach. *Biochimie*. 73:1295–1302.
- Mayer, C., K. Gröbner, K. Muller, K. Weisz, and G. Kothe. 1990. Orientation-dependent deuterium spin-lattice relaxation times in bilayer membranes: characterization of the overall lipid motion. *Chem. Phys. Letters*. 165:155–161.
- McMullan, R. K., T. F. Koetzle, and M. D. Fronckowiak. 1992. Structure of  $[20\text{-CD}_3]$ ,  $[20\text{-CD}_3]$ -methylpregnene-3, 20-diol methanolate from neutron diffraction at 123 K. *Acta Crystallogr.* 48:1509–1512.
- McMullen, T. P. W., A. H. Lewis, and R. N. McElhaney. 1993. Differential scanning calorimetric study of the effect of cholesterol on the thermotropic phase behavior of a homologous series of linear saturated phosphatidylcholines. *Biochemistry*. 32:516–522.
- McMullen, T. P. W., and R. N. McElhaney. 1995. New aspects of the interaction of cholesterol with dipalmitoylphosphatidylcholine bilayers as revealed by high-sensitivity differential scanning calorimetry. *Biochim. Biophys. Acta*. 1234:90–98.
- McMullen, T. P. W., and R. N. McElhaney. 1996. Physical studies of cholesterol-phospholipid interactions. *Curr. Opin. Colloid Interface Sci.* 1:83–90.
- Pott, T., J. C. Maillet, and E. J. Dufourc. 1995. Effects of pH and cholesterol on DMPA membranes: a solid state  $^2\text{H}$ - and  $^{31}\text{P}$ -NMR study. *Biophys. J.* 69:1897–1908.
- Press, W. H., S. A. Teukolsky, W. T. Vetterling, and B. P. Flannery. 1992. Numerical Recipes in Fortran, 2nd ed. Cambridge University Press, Cambridge.
- Sanders, C. R., and J. H. Prestegard. 1991. Orientation and dynamics of  $\beta$ -dodecyl glucopyranoside in phospholipid bilayers by oriented sample NMR and order matrix analysis. *J. Am. Chem. Soc.* 113:1987–1996.



- Sawick, P., and B. M. Craven. 1980. The structure of cholesteryl laurate at 198K. *Acta Crystallogr.* 36:3027–3033.
- Stankovic, S., D. Miljkovic, L. Medic-Mijacevic, K. Gasi, and C. Courseille. 1994. 17 $\alpha$ -benzyl-3 $\beta$ , 17 $\beta$ -dihydroxy-5-androstene. *Acta Crystallogr.* 50:312–314.
- Stockton, G. W., and C. P. Smith. 1976. A deuterium nuclear magnetic resonance study of the condensing effect of cholesterol on egg phosphatidylcholine bilayer membranes. I. Perdeuterated fatty acid probes. *Chem. Phys. Lipids.* 17:251–263.
- Taylor, M. G., T. Akiyama, and I. C. P. Smith. 1981. The molecular dynamics of cholesterol in bilayer membranes: a deuterium NMR study. *Chem. Phys. Lipids.* 29:327–339.
- Watson, D. F. 1982. ACORD—Automatic contouring of raw data. *Computers & Geosciences* 8:97–101.
- Weeks, C. M., P. D. Strong, W. L. Duax, and L. E. Vickery. 1983. 17 $\alpha$ -phenyl-5-androstene-3 $\beta$ ,17 $\beta$ -diol-Cyclohexane (2:1), C<sub>25</sub>H<sub>34</sub>O<sub>2</sub>.1/2C<sub>6</sub>H<sub>12</sub>. *Acta Crystallogr.* 39:1698–1703.
- Weisz, K., G. Grobner, C. Mayer, J. Stohrer, and G. Kothe. 1992. Deuteron nuclear magnetic resonance study of the dynamic organization of phospholipid/cholesterol bilayer membranes: molecular properties and viscoelastic behavior. *Biochemistry.* 31:1100–1112.
- Worcester, D. L., and N. P. Francks. 1976. Structural analysis of hydrated egg lecithin and cholesterol bilayers. II. Neutron diffraction. *J. Mol. Biol.* 100:359–378.